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Regional Specific Brain ApoE Modulation by the Estrous Cycle and Exogenous Estrogen

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Regional Specific Brain ApoE Modulation by the estrous cycle and exogenous estrogen

BY:

Emily Rosario

THESIS

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I HEREBY RECOMMEND THAT THIS THESIS BE ACCEPTED AS FULFILLING THIS PART OF THE GRADUATE DEGREE CITED ABOVE

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ACKNOWLEDGEMENTS

The past two years have been a wonderful journey which brought me to love the world of research. I would like to thank my wonderful mentor Dr. Nathan. His support, encouragement, guidance, and assistance throughout my research has allowed me to accomplish a great deal. I would also like to thank my committee members, Dr. Kipp Kruse and Dr. Kip McGilliard for their assistance and support. I would like to thank Dr. Scott Meiners for his assistance with SPSS. I would like to thanks the Graduate School for supporting my as a research assistant. I would also like to thank Dean Augustine for his support. I would like to say a very special thank you to my labmates Matt Kircher, Fei Shen, Anna Barsukova, Melissa Litherland, Nick Short, Paul McAdamis, and Aubrey Osborne who have been supportive, helpful and above all wonderful friends. Last but not least, I would like to say thank you to my family and fiancé Nick for their support, encouragement, and love which helped me to continue through everything. I hope that this research will prove useful in the struggle to unravel the mechanisms leading to Alzheimer's Disease.

TABLE OF CONTENTS

List of Figures

Abstract

Previous studies have demonstrated that 17β-estradiol can modify apolipoprotein E (apoE) expression. I found that apoE protein varied as a function of the estrous cycle and 17β-estradiol (E2) in a region specific manner in the mouse brain. I also found that apoE concentration was lowest on estrus in the hippocampus, cingulate cortex and frontal cortex; apoE concentration was highest on estrus in the olfactory bulb and cerebellum. No changes in apoE were found in the striatum throughout the estrous cycle. Exogenous E2 significantly raised tissue levels in the olfactory bulb and cerebellum, but did not increase apoE in cortical samples nor in the striatum. E2 elevates levels of apoE in a region- and cycle day-specific manner that may be related to the estrogen receptor subtype in that area.

Introduction

Apolipoprotein E (apoE) is a 299 amino acid component of lipoproteins with a molecular weight of 34-kDa. ApoE plays a vital role in the regulation of lipoprotein metabolism and in the control of lipid transport and lipid redistribution among target tissues and cells (Mahley, 1988, Weisgraber, 1994). Lipid transport and redistribution is regulated by apoE via interaction with lipoprotein receptors (Mahley, 1988). Cellular uptake and degradation of the lipoproteins is initiated by receptor-lipoprotein binding. The lipid becomes available for utilization in the regulation of intracellular cholesterol metabolism. ApoE, therefore, serves as a ligand for the receptor-mediated clearance of lipoproteins from the plasma (Rall et al., 1982).

The ApoE Gene:

An 1163-nucleotide mRNA encodes apoE. The apoE gene is 3597 nucleotides in length and contains four exons (Mahley, 1988). In humans, apoE is a polymorphic protein, existing as three common isoforms (apoE2, apoE3, and apoE). These are products of the three alleles (designated ε2, ε3, and ε4 respectively) located at a single gene locus on the long proximal arm of chromosome 19 (Holtzman, 2000). The molecular basis of this polymorphism of the apoE gene results from cysteine-arginine interchanges at two positions in the apoE protein (Weisgraber, 1994). These single amino acid substitutions are found at residues 112 and 158 (Rall, 1982). The most common isoform, apoE3, contains cysteine at residue 112 and arginine at position 158. ApoE2 has cysteine at both positions and apoE4 contains arginine at both positions (Weisgraber, 1994).

ApoE Synthesis and Function:

The largest quantity of apoE is synthesized in the liver followed by the brain and many other organs (Boyles, 1985). In these organs, a wide variety of cell types are capable of

producing apoE, which includes oligodendrocytes (Stoll., 1989), astrocytes (Pitas, 1987), and macrophages (Mahley, 1988). In addition to its previously mentioned function of lipid metabolism, apoE has also been implicated in a wide variety of other physiological processes throughout the body. ApoE may have a structural role in a number of lipoprotein particles as well as regulating their metabolism (Wisniewski and Frangione, 1992). ApoE is also thought to function in immunoregulation (Cuthbert and Lipsky, 1984), nerve growth, nerve repair and regeneration in both the central nervous system (CNS) and the peripheral nervous system (PNS), (Ignatius, 1986; Ignatius, 1987; Snipes, 1986; Boyles, 1989; Handelmann, 1992), modulation of intracellular cholesterol utilization (Reyland, 1991), steroidogenesis in adrenal cells (Reyland and Williams, 1991), and as an activator or modulator of hepatic lipase (Ehnholm, 1984; Landis et al., 1987; Thurin et al., 1991, 1992). ApoE is thought to participate in the regeneration of peripheral nerves after injury (Boyles et al., 1989), and in the mobilization and redistribution of lipids during normal development of the nervous system (Pitas et al., 1987).

ApoE in the Nervous System:

The exact function of apoE in the nervous system is poorly understood. Injury in either the CNS or PNS results in an increase in apoE levels (Ignatius et al., 1986). Following peripheral nerve injury in rats, the synthesis of apoE increases by 250- to 350-fold within three weeks. (Snipes et al., 1986; Boyles et al., 1989). It has also been reported that macrophages synthesize and release apoE following peripheral nerve lesion, which accumulates, to 5% of total extracellular protein (Skene and Shooter, 1983). Studies suggest that the purpose of this accumulation of apoE is to scavenge cholesterol from the degenerating myelin and recycle it to the growth cones of sprouting axons for membrane biosynthesis (Mahley, 1988; Goodrum et al., 1995; Poirier et al., 1993).

Based on these observations, it has been proposed that apoE is involved in neurodegenerative processes by isoform-specific effects on cytoskeletal stability and neurite outgrowth (Mahley et al., 1995; Weisgraber and Mahley, 1996). *In vitro* studies with dorsal root ganglion neurons have shown that addition of apoE3 to a culture stimulated neurite outgrowth whereas apoE4 decreased neurite extension (Nathan et al., 1994). These data imply that apoE is important for peripheral nerve regeneration (Mahley, 1988). The data from apoE knockout (apoE KO) mice, however, does not support this hypothesis. Regenerating nerves in both control mice and apoE KO mice were morphologically identical at two and four weeks following sciatic nerve crush (Popko et al., 1993; Goodrum, 1995). This suggests that other apolipoproteins in the PNS may substitute for apoE when it is absent. Hence, the specific role of apoE and its importance in the PNS remains unclear.

ApoE is the principle apolipoprotein in the brain and cerebrospinal fluid (CSF). However the precise function and mechanism of action of apoE is even less clear in the central nervous system. The majority of apoE in the CNS is synthesized and secreted primarily by glial cells, followed by microglia (Pitas et al., 1987; Pitas et al., 1987; Borghini et al., 1995; Boyles et al., 1985; Naikai et al., 1996). ApoE is the only apolipoprotein in the CNS that is able to interact with lipoprotein receptors (Pitas et al., 1987; Borghini et al., 1995). Cells within the brain express four receptors for apoE-containing lipoproteins: the low-density lipoprotein (LDL) receptor, the LDL receptor-related protein (LRP), the very low density lipoprotein (VLDL) receptor, and the glycoprotein (gp) 330. The LDL receptor and the LRP are expressed by neurons (Pitas et al., 1987; Boyles et al., 1985). It has been shown that the VLDL receptor in the CNS is expressed in some human neurons, whereas gp330 is expressed by brain ependymal cells (Willnow et al., 1992; Sakai et al., 1994; Kim et al., 1996; Kounnas et al., 1994). It has been

reported that human apoE-containing lipoproteins bind to fibroblast LDL receptors and that the LDL receptor and the LRP mediate the binding and internalization of apoE-containing lipoproteins in cultured neurons (Bellosta et al., 1995). These studies provide evidence that the apoE and apoE-containing lipoproteins are present within the brain where they can interact with neurons and that lipoprotein transport by apoE is important for normal functioning of adult neurons.

Increased apoE immunoreactivity is present in the brains of patients with such neurological disorders as Down's syndrome, Creutzfeld-Jacob disease, and Alzheimer's disease (AD) (Namba et al., 1991). It has been demonstrated that expression of apoE increases following optic nerve injury, but absolute levels of apoE do not increase (Ignatius et al., 1986). ApoE mRNA is increased in the brains of AD patients (Diedrich et al., 1991) and in response to injury in both the CNS (Boyles et al., 1989) and PNS (Snipes et al., 1986).

It has been shown that addition of apoE4 to a culture inhibits neurite extension, whereas apoE3 stimulates neurite outgrowth in transformed murine neuroblastoma (Neuro-2a) cells (Bellosta et al., 1995). Recent studies show apoE KO mice display disruption of the dendritic cytoskeleton and significant synaptic loss with age they also show a reduced recovery following perforant pathway lesioning (Masliah et al., 1995; Masliah et al., 1996; Masliah et al., 1997). Synaptophysin (a marker for presynaptic terminals) and microtubule-associated protein (MAP-2, a dendritic marker) levels in the hippocampus and neocortex of apoE KO mice were shown to decrease as compared to age-matched control mice. However, other studies have not observed any significant morphological deficits in apoE KO mice (Anderson et al., 1998). The reasons underlying these inconsistencies are not clear, but differences in the strain and age of both the apoE KO and the control mice used may have contributed to the inconsistent results observed.

In contrast to the morphological studies, behavioral studies have consistently shown that apoE KO animals exhibit spatial learning deficits (Masliah et al., 1996; Gordon et al., 1995; Gordon et al., 1996). Infusion of recombinant apoE into the lateral ventricles of apoE KO mice reversed behavioral and morphological anomalies (Masliah et al., 1996). Other studies involving apoE KO mice have suggested that apoE is involved in protecting the brain against acute injury (Chen et al., 1997). These results provide convincing evidence that apoE plays a critical role in preservation, neuroprotection, and plasticity within the CNS.

Alzheimer's Disease

By studying apoE within the CNS some questions have been answered about neurodegenerative disorders, especially Alzheimer's disease (AD). The apoE genotype has been shown to be a major risk factor for AD. ApoE immunoreactivity is associated with the characteristic pathological structures present in the brains of AD patients, neurofibrillary tangles and neuritic plaques (Namba et al., 1991; Wisniewski and Frangione, 1992; Schmechel et al., 1993; Strittmatter et al., 1993). Neurofibrillary tangles are intracellular and contain structures known as paired helical filaments (Goedert et al., 1992). The neuritic plaques are, for the most part, extracellular and constitute classical amyloid deposits and often a neuritic component. The major protein present in these plaques is the amyloid beta peptide (Ab), which is formed by cleavage of the amyloid precursor protein (APP) (Haass and Selkoe, 1993). Studies have shown that apoE is associated with Ab deposits in neuritic plaques and in the angiopathy of cerebral vessels (Strittmatter et al., 1993). The role of these plaques and tangles in the progression or onset of AD is not yet clear.

There are two types of AD: familial Alzheimer's disease (FAD), and late-onset sporadic. FAD represents approximately 5% of patients and usually occurs before the age of 65, whereas

late-onset AD accounts for a majority of AD cases and occurs after the age of 60 (Digivanna, 2000). Recent studies have indicated a relationship between the apoE4 allele and late-onset AD (Strittmatter, 1993). It has been demonstrated that the risk of early-onset AD and disease progression increase is related to the number of apoE4 alleles in a dose-dependent fashion (Tsai et al., 1994; Corder et al., 1993). The frequency of the apoE4 allele has been greatly over-represented in late-onset AD patients (representing 52% of the subjects) versus controls (16%), and the risk of AD in individuals homozygous for the apoE4 allele is over five times that of homozygous apoE3 individuals (Corder et al., 1993).

Another recent study suggests that the apoE4 allele may also be involved with age of onset in Parkinson's disease. This evidence strongly suggests that there is a definite correlation between apoE polymorphism and the development of neurodegenerative disorder (Nisar, 1999). The mechanism behind the pathogenesis of these disorders and the exact effects of apoE on CNS neurons remain unclear. One possible mechanism of apoE in AD may involve neuronal plasticity, based on previous studies, which suggest that apoE may play a crucial role in nerve regeneration.

AD and Estrogen:

In addition to apoE genotype, it seems that estrogen plays an important protective role in the development of AD. Estrogen is a hormone dominant in the female reproductive system. Estrogen can improve blood circulation in the brain as well as stimulate nerve cell growth. It has also been shown to increase levels of acetylcholine, and it impedes the deposition of β-amyloid (Inestrosa et al., 1998). The risk of AD and related dementia for women who used estrogen replacement therapy (ERT) was reduced by about one third below that of women who had never used ERT (Paganini-Hill and Henderson, 1996). Recent studies have suggested that estrogen's

protective effects are through its action as a trophic factor for cholinergic neurons, a modulator for the expression of apoE in the brain, or an antioxidant compound decreasing the neuronal damage caused by oxidative stress (Inestrosa et al., 1998).

As seen earlier it has been demonstrated that ERT increases cerebral and cerebellar blood flows in postmenopausal women (Ohkura et al., 1995). Cerebral blood flow values are shown to be higher in females than in males until the age of 50 (Davis et al., 1983) or 60 (Shaw et al., 1984). Furthermore, ERT improves cognitive functions and increases regional cerebral blood flow in female patients with dementia of the Alzheimer type (Ohkura et al., 1994).

Estrogen, glial cells and ApoE

It has been clearly shown that estrogen replacement therapy (ERT) reduces the risk of AD and also delays the age of clinical onset (Waring et al., 1999; Paganini-Hill, 1994). It has also been found that estrogen replacement in experimental animals promotes recovery from neurological damage (Stone et al., 1998; Stone et al., 1997; Toung et al., 1998), and that estrogen administration increases apoE and glial markers. The exact mechanism by which estrogen facilitates nerve repair is still unknown. As shown above both astrocytes and microglia produce apoE. Estrogen appears to regulate apoE gene expression in an organ specific manner (Srivastava et al., 1996). Studies have shown that estrogens effects on glia represented an interaction between neurons and glial cells (Stone et al., 1998).

There is a great deal of emerging evidence that ERT both reduces AD and slows cognitive decline in women (Stone et al., 1998). Parallel studies in mice, evaluating the effects of long-term estrogen elevation, found only transient changes in the synaptic density of olfactory neurons (Nathan, personal communication). These results suggest that estrogen's effects on the CNS, in neurologically intact animals, are only transitory.

Estrogen Receptors

Estrogen receptors have been reported in astroglia, sheathing glia, and microglia (Azcoitia et al., 1999; Mor et al., 1999). Until recently it was thought that the actions of estrogen were mediated by a single nuclear estrogen receptor. Studies have found that there are two types of estrogen receptors, the classic ER-α, and the novel ER-β (Shughrue et al, 1997). Neurons of the olfactory bulb, cerebellum, and areas of the hypothalamus show exclusively ERβ. Both receptor types are found throughout the rostral-caudal extent of the brain. The region specific expression of ER-α and ER-β is proving to be important in determining the physiological responses of neuronal populations to estrogen action (Shughrue et al, 1997).

Aim of the Study

The objectives of this study were to measure brain region specific concentrations of apoE protein during the estrous cycle in the mouse and to determine if exogenous E2 could alter levels of apoE protein. My hypothesis is that E2 will modulate apoE levels in a region specific manner. The rationale behind this hypothesis is the differential localization of ERα, and ERβ.

Materials and Methods

Mice / Treatment groups

For this study I used control female mice (Jax control) that were 2-3 months in age. For the estrous cycle experiment the estrus phase was the treatment group. For the exogenous estradiol experiment the treatment groups included; ovariectomized /Estrogen pellet /Saline irrigated and ovariectomized /Placebo pellet /Saline irrigated. Males were used as a comparison in this study.

Vaginal smears

Normal, female mice approximately 2-3 months old at the start of the study were evaluated by vaginal smears for at least three weeks to assure normal estrous cycles. The mice were smeared with 1X PBS using a glass Pasteur pipette. Examination of the smears using a phase contrast microscope led to the identification of cell types. Nucleated epithelial cells signify proestrus, cornified epithelial cells signify estrus and leukocytes are found in diestrus.

Surgery

Animals were either ovariectomized (OVX) or sham operated, depending on the treatment group. All animals were anesthetized with an intraperitoneal injection of sodium pentobarbital (80 mg/kg). The incision site was shaved and an aseptic bilateral dorsal incision made just under the rib cage. The fallopian tube was clamped just below the ovary and the ovary was then completely removed. The muscle layer was sewn with silk ligatures and the skin was closed with auto clips. The same procedure was followed on the opposite side. In the sham operation the same procedure was followed except the ovary was not removed.

Sacrifice

Control female mice were sacrificed on the following days of the estrus cycle, proestrus, estrus, and diestrus. Mice were anesthetized by an intraperitoneal injection of sodium pentobarbital (80mg/kg). After the animals were deeply anesthetized, a needle was inserted transcardially and the animals were thoroughly perfused with phosphate buffered saline (pH 7.4). The brain was removed from the skull case and the following areas were dissected using visual guidance: 1) olfactory bulb; 2) frontal cortex dorsal to the rhinal fissure; 3) cingulated cortex; 4) hippocampus; 5) striatum; and 6) cerebellar cortex. In addition three males were prepared similar to the females.

Tissue Preparation

The samples were homogenized in ice cold TMN buffer (25 mM Tris-HCl [pH 7.6] 3 mM $MgCl_2$, 100 mM NaCl, 1 mM phenylmethylsulfonyl fluoride) (Xu et al., 1996). The homogenate was lysed by adding 1% Triton X-100, 0.5% deoxycholate, and 0.2% SDS on ice for 5 min (Xu et al., 1998). The homogenate was then centrifuged for 2 min in a microcentrifuge (g = 13,000) (Xu et al., 1998). The supernatant was saved for protein assay and western immunoblot analysis.

Protein Assay

Protein assay was performed by BCA protein assay method as described by the manufacturer (Pierce). Briefly, 1 µl of homogenized sample was diluted to 99 µl with double distilled water, and 30 µl of diluted samples (in triplicates) were assayed.

SDS – Polyacrylamide Gel Electrophoresis

Proteins in the olfactory bulb were resolved by SDS-PAGE as previously described (Bellosta et al., 1995). Briefly, 20 µg of sample protein was mixed with an

equal volume of 2X Lammeli sample buffer (6.25 ml 4X Tris/SDS [pH 6.8], 5 ml glycerol, 1g SDS, 0.5 ml 2-mercaptoethanol, bromophenol, 13.25 ml dH_2O). Samples were boiled for 5 min and then centrifuged at 14,000 g for 5 min. The gel cassettes were inserted into the buffer tank of an EC120 Mini gel vertical system (E-C Apparatus Corporation, St. Petersburg, FL) containing 1X running buffer, pH 8.3 [250 ml of 5X running buffer (15 g Tris-base, 72 g glycine, 5 g SDS, 750 ml dH_2O].

The samples and 5 µl of kaleidoscope prestained standards (161-0324, Bio-Rad Laboratories, Hercules, CA) were electrophoresed through a pre-cast 4-20% gradient gel (Fisher, FB3435). Samples were electrophoresed at 80 volts until separation began, and then at 140 volts until the dye front reached the bottom of the gel.

Protein Transfer

Following electrophoresis, the gel was placed in transfer buffer (3.03 g Tris-base, 14.4 g glycine, 200 ml methanol, 800 ml dH_2O) on a shaker. The transfer membrane (Immobilon-P IPVH00010, Millipore, Bedford, MA) was soaked in methanol for 5 sec and then washed in dH_2O for 5 min. The gel was placed on presoaked filter paper in the holder and the transfer membrane was placed on top of the gel. Using a trans-blot transfer cell (170-3930, Bio-Rad), proteins from the gel were transferred onto the membrane by passing 100 volts for an hour.

Western Blotting

Apolipoprotein E

ApoE was quantified as previously described (Xu et al., 1996). Briefly, the blots were incubated in polyclonal goat antiserum against human apoE (178479, Calbiochem, San Diego, CA) (1:5,000 dilution in T-TBS [pH 7.6] 0.1M Tris, 0.15M NaCl, 0.1%

Tween-20) for 30 min on a shaker at room temperature. The membrane was then washed 5 times (10 min each) in T-TBS. The blot was then incubated in the secondary antibody solution (rabbit anti-goat IgG-HRP [AP106P, Chemicon, Temecula, CA] 1:10,000 dilution) for 30 min on a shaker at room temperature. Blots were washed with T-TBS 6 times (10 min each) (Xu et al., 1996). Immunoreactive bands were then visualized with SuperSignal West Pico Chemiluminescent substrate (34080, Pierce, Rockford, IL) and then exposed to BioMax film (Kodak).

Quantitation/Data Analysis

All experiments were repeated at least three times to assure reproducibility of the results. Bands were quantified by densitometry (Scion Image). As an internal control, the blots also contained an olfactory bulb extract from unlesioned control female animals.

Statistical analysis of the immunoblot data was by a repeated measures analysis of variance (Systat 10) with region as the repeated measure and the part of the estrus cycle as the independent measure. When a statistically significant interaction between region and condition was found, we performed a one-way analyses of variance to further specify the character of the significant differences found in the initial analysis.

Results:

In this study, female mice 2-3 months old were evaluated by vaginal smears for at least three weeks to assure normal estrous cycles. Groups of three mice were sacrificed on diestrus, proestrus, and estrus. The brain was removed from the skull case and the following areas were dissected using visual guidance: 1) olfactory bulb; 2) frontal cortex dorsal to the rhinal fissure; 3) cingulated cortex; 4) hippocampus; 5) striatum; and 6) cerebellar cortex. In addition three males were prepared similar to the females. Statistical significance was found for estrus ($F=17:01$, $df=2,6$; $p<0.003$), region ($F=8.57$; $df=5,30$; $p<0.001$) and for the interaction between cycle and regions ($F=9.60$;$df=10,30$; $p<0.001$). The simple main effects of estrus and regions showed that total apoE regional concentrations of apoE were higher on proestrus and diestrus than on estrus. Olfactory bulb showed the highest concentration and cerebellum the lowest irrespective of cycle stage. However the significant interaction of estrus by region limits any interpretation of simple main effects.

Post-hoc analysis with one-way analysis of variance found that the hippocampus ($F=13.57$, $df=2,6$; $p<0.006$), cingulate ($F=14.83$, $df=2,6$; $p<0.005$) and frontal ($F=25.95$, $df=2,6$; $p<0.001$) showed minimum apoE expression on estrus and roughly comparable levels during proestrus and diestrus (figures 1,2,&3). Conversely, in both olfactory bulb ($F=61.00$, $df=2,6$; $p<0.001$) and cerebellum ($F=39.00$ $df=2,6$; $p<0.001$) peak levels of apoE occurred during estrus (Figures 4&5). The caudate-putamen complex showed no differences as a function of estrus cycle ($F=0.139$, $df=2,6$; $p<0.873$)(Figure 6). Analysis of the regional data from males ($n=3$) did not find statistical significance among brain regions ($F=1.659$; $df=5,10$; $p<0.232$). However, the same regional pattern was obtained

as in females. The olfactory bulb had the highest concentration of apoE, the cerebellum lowest and cortical areas were intermediate. The small sample size for males (n=3) probably resulted in non-significance. The apparent difference between males and females in total apoE could not be analyzed as immunoblots were performed a disparate time.

Exogenous E2 increased apoE in OVX mice. We found a significant **E2** effect ($F=28.85$; $df=1,4$; $p<0.006$), a significant region effect ($F=39.213$; $df=5,20$; $p<0.001$) and a significant region by E2 interaction ($F=3.789$; $df=5,20$; $p<0.014$) in the initial analysis of variance. E2 replacement resulted in a 68% increase in overall apoE. OB had the highest levels of apoE. In contrast to the previous analysis, the cingulate showed the lowest levels of apoE pooled over the two E2 conditions. However, the presence of a significant region by E2 interaction limited further conclusions. Post-hoc analysis of the interaction, with a one-way ANOVA, found a 74% increase of apoE with E2 in olfactory bulb ($F=23.90$; $df=1,4$; $p<0.008$)(Figure 7) and a 110% increase in cerebellum ($F=48.40$; $df=1,4$; $p<0.002$)(figure 8). E2 effects in hippocampus ($F=4.70$; $df=1,4$; $p<0.096$), frontal ($F=1.78$; $df=1,4$; $p<0.253$) and cingulate ($F=1.58$; $df=1,4$; $p<0.277$) did not reach statistical significance(Figures 9,10,11). Caudate showed no effect of E2 ($F=0.295$; $df=1,4$; $p<0.616$)(figure 12).

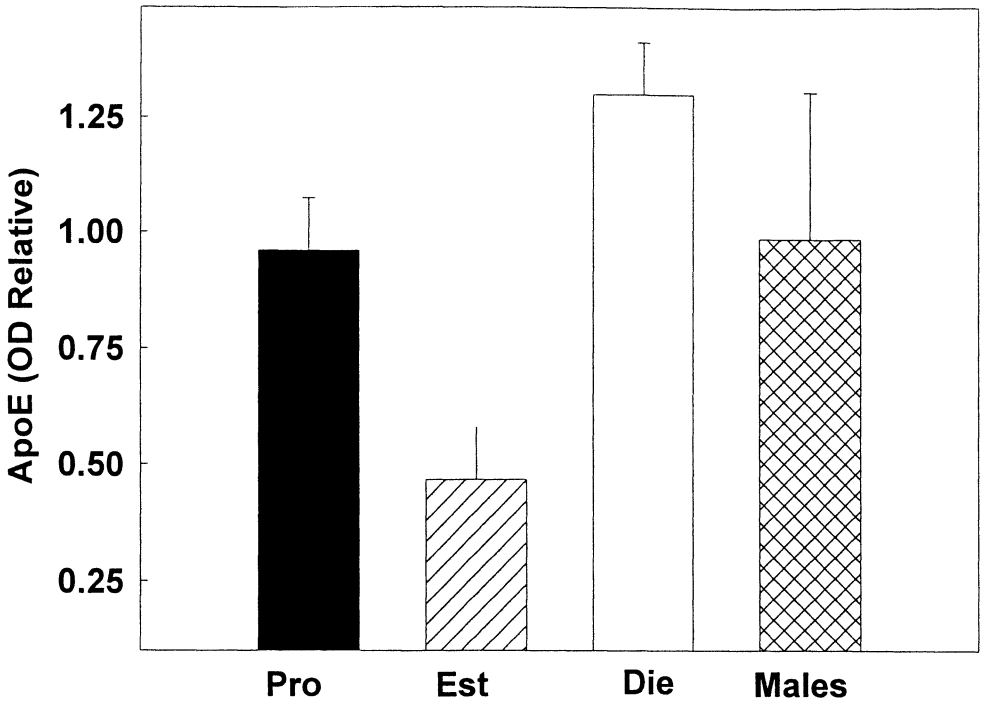

Figure 1: ApoE concentrations (mean ± SE) in hippocampus during the estrous cycle. Significant differences are seen during estrus when compared to the other phases. Males, which show levels of apoE similar to proestrus are included for comparison.

Figure 2: ApoE concentrations (mean ± SE) in the cingulate and the estrous cycle. Proestrus and diestrus have approximately equal levels of apoE while estrus has significantly less. Males are included for comparison and have apoE levels similar to proestrus and diestrus.

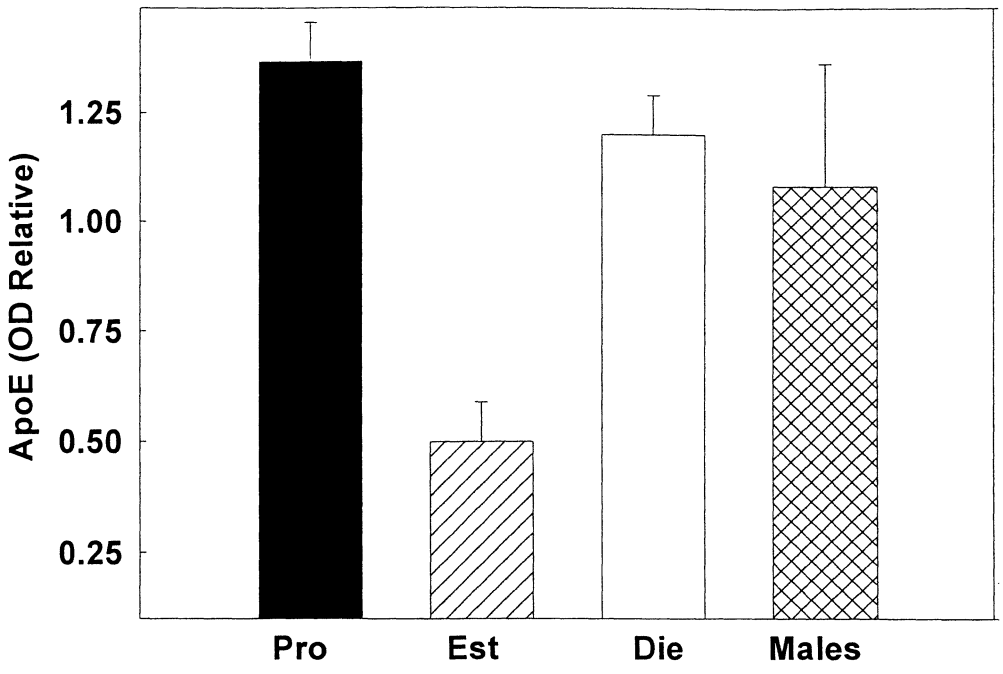

Figure 3: ApoE concentrations (mean ± SE) in the frontal lobe and the estrous cycle. Levels of apoE are highest in proestrus and diestrus followed by estrus. Males are included for comparison and have apoE levels similar to diestrus.

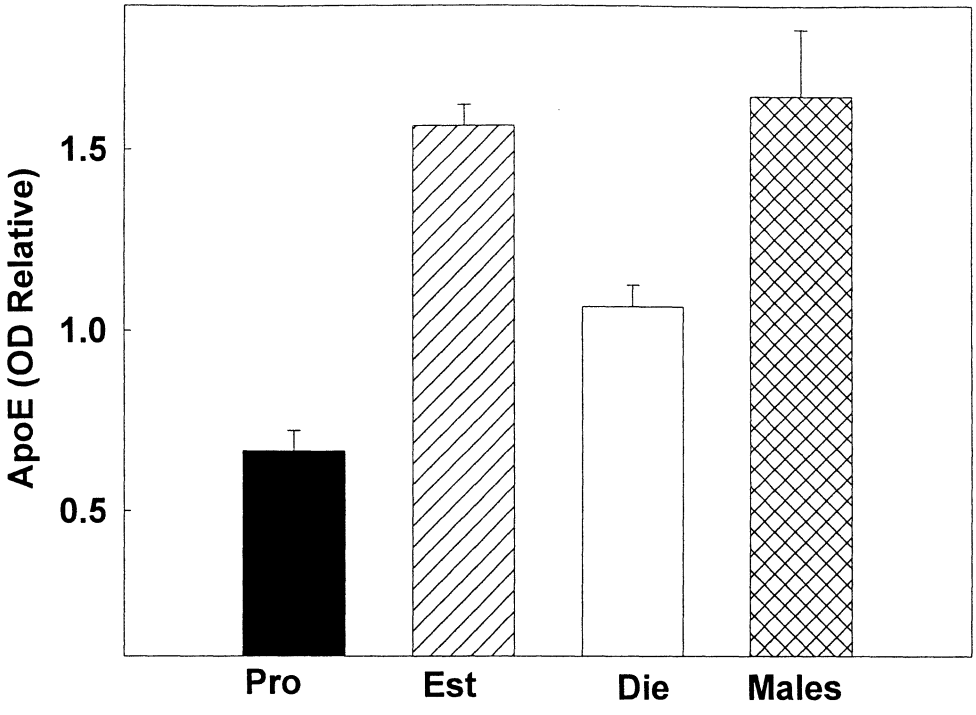

Figure 4: ApoE levels (mean ± SE) in the olfactory bulb and estrous cycle. Estrus had the highest levels of apoE followed by diestrus and proestrus. Males are included for comparison and have apoE concentrations comparable to estrus.

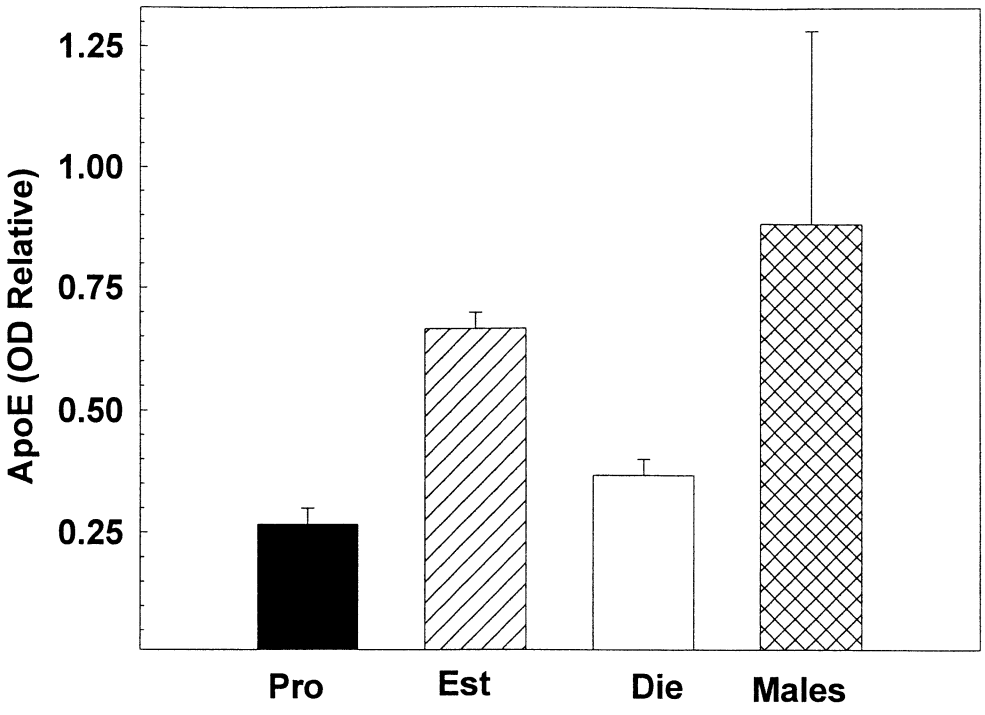

Figure 5: ApoE levels (mean ± SE) in the cerebellum during the estrous cycle. ApoE levels are highest in estrus followed by proestrus and diestrus. Males are included for comparison and have apoE levels similar to estrus.

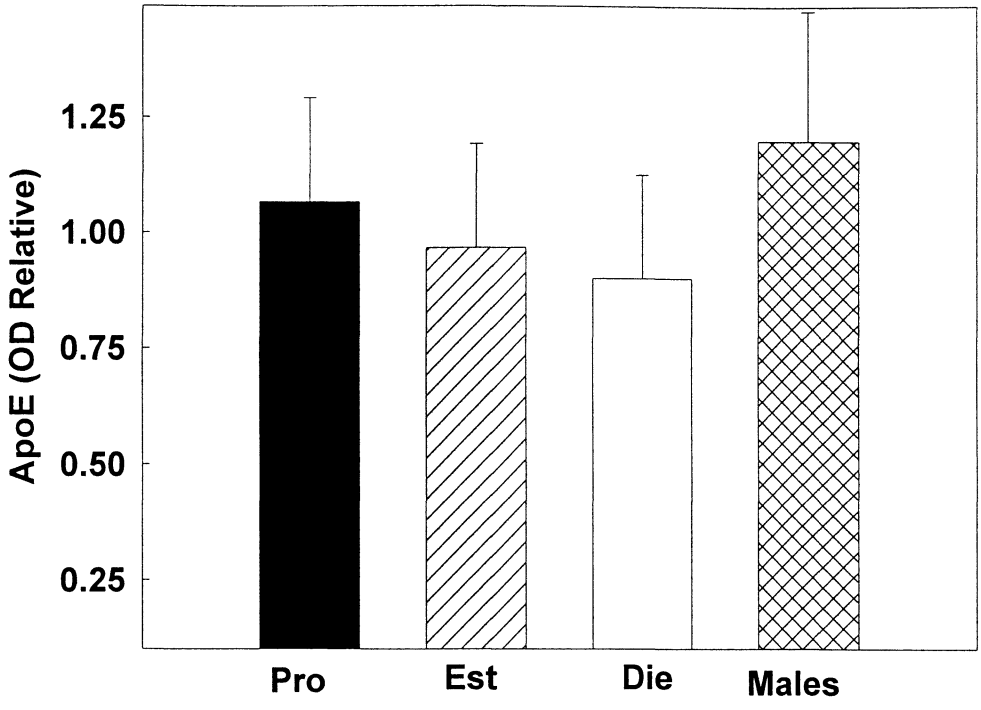

Figure 6: ApoE levels (mean ± SE) in the caudate putamen (striatum) during the estrous cycle. ApoE levels are approximately the same for all three stages. Males are included for comparison.

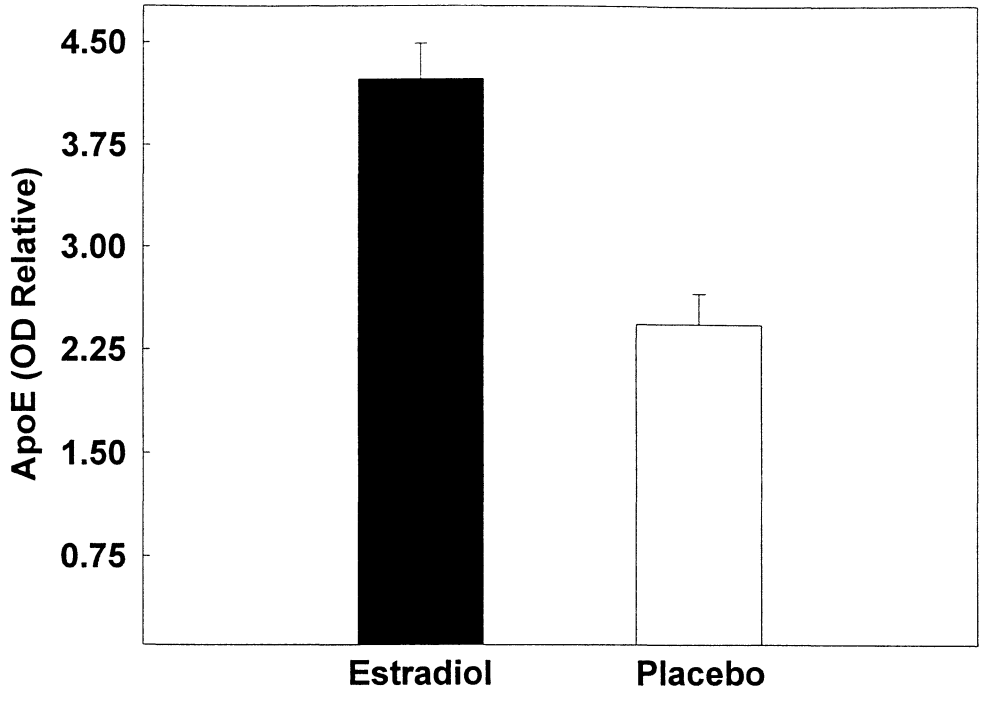

Figure 7: 17β-estradiol effects on apoE levels (mean ± SE) in the olfactory bulb. When compared to placebo, 17β-estradiol significantly increased apoE concentrations in the olfactory bulb.

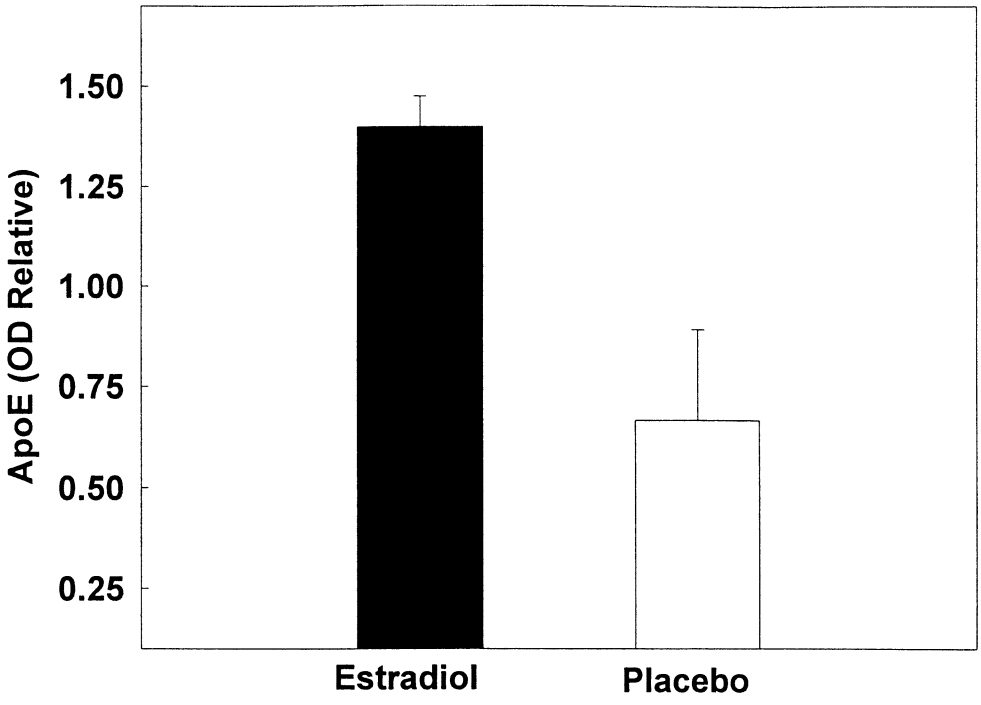

Figure 8: 17β-estradiol effects on apoE levels (mean ± SE) in the cerebellum. When compared to placebo, 17β-estradiol significantly increased apoE concentrations in the cerebellum.

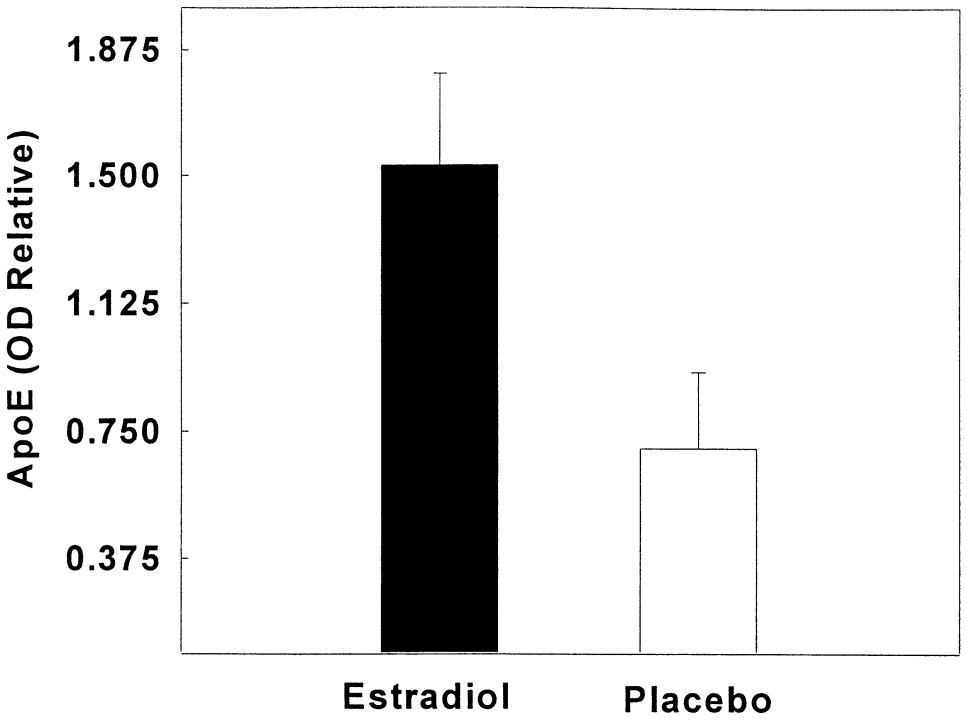

Figure 9: 17β-estradiol effects on apoE levels (mean ± SE) in the hippocampus. No significant effects were found between placebo and 17β-estradiol treated groups.

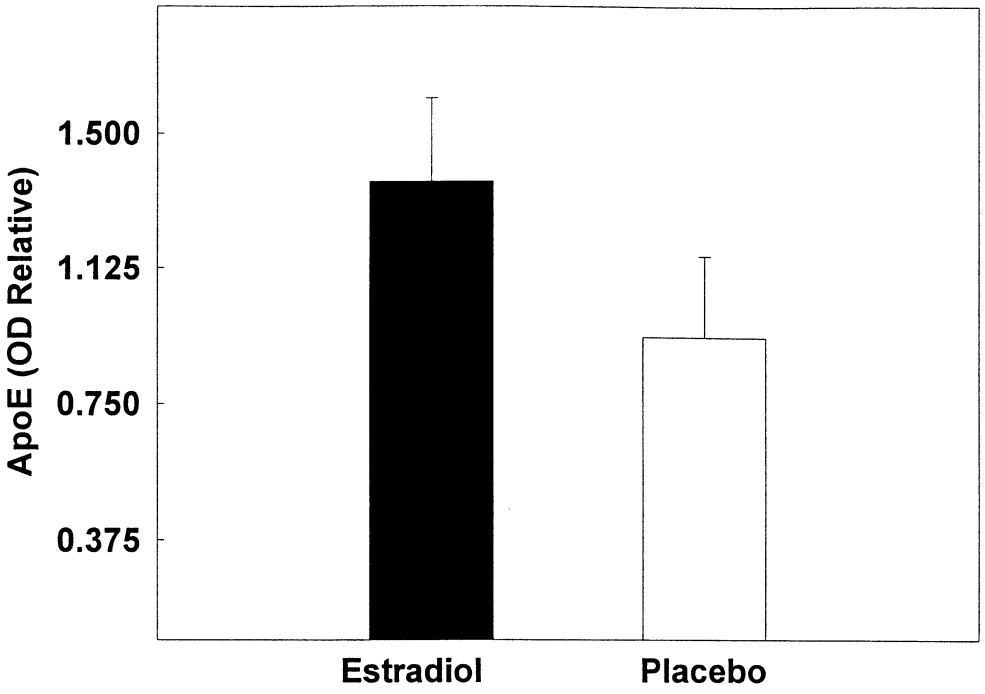

Figure 10: 17β-estradiol effects on apoE levels (mean ± SE) in the frontal lobe. No significant effects of were found between placebo and 17β-estradiol treated groups.

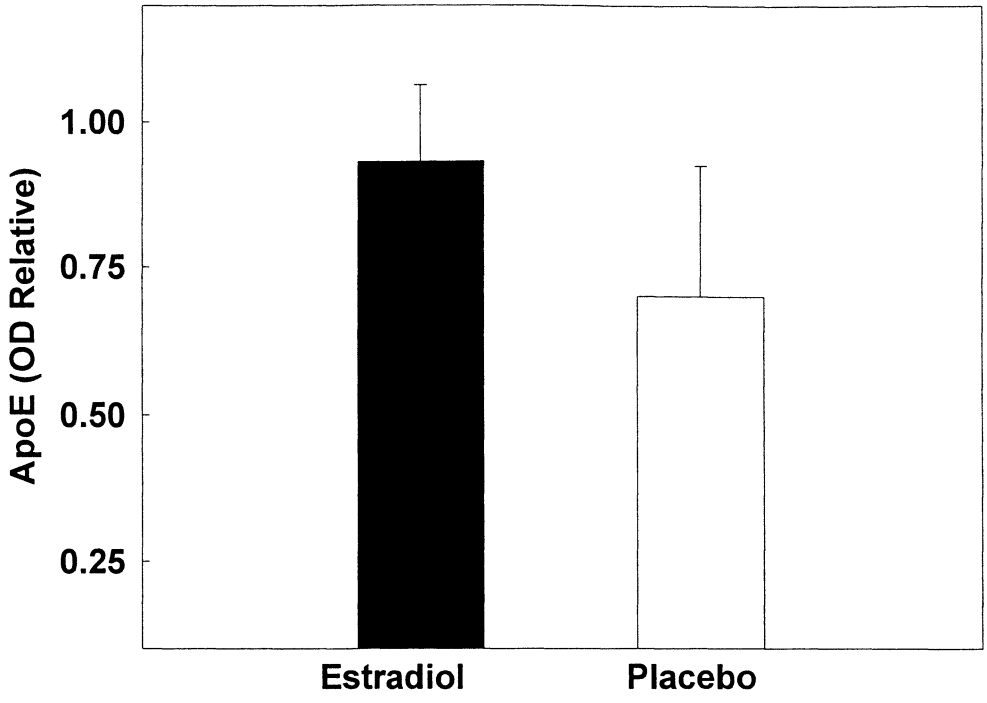

Figure 11: 17β-estradiol effects on apoE levels (mean ± SE) in the cingulate. No significant effects were found between placebo and 17β-estradiol treatment groups.

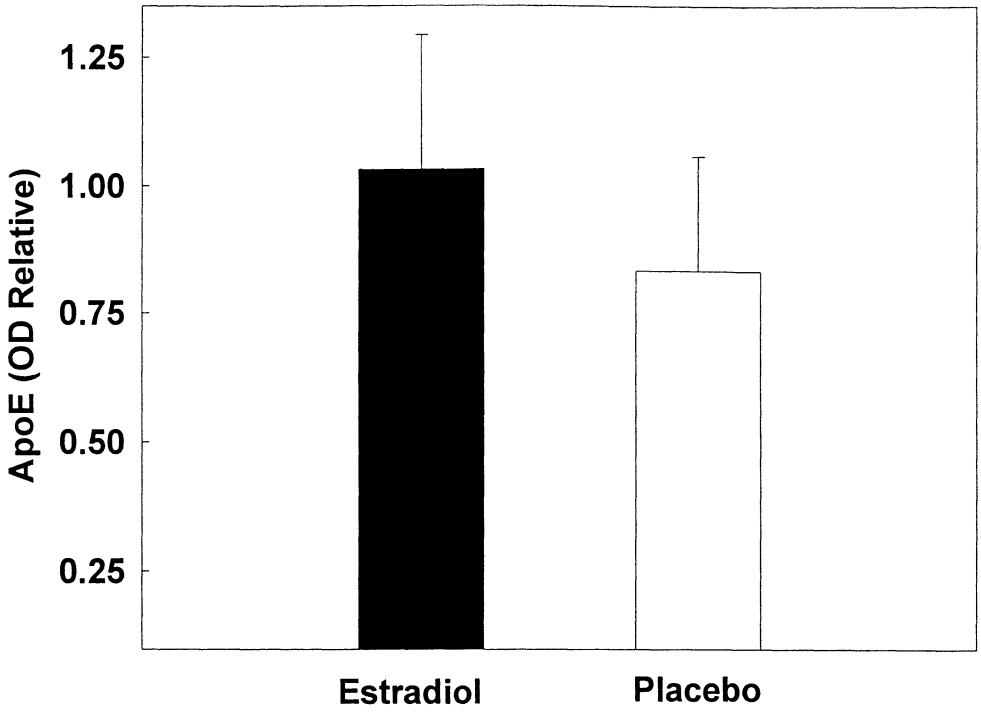

Figure 12: 17β-estradiol effects on apoE levels (mean ± SE) in the caudate putamen (striatum). No significant effects were found between placebo and 17β-estradiol treated groups.

Discussion:

Previous studies on the effects of lesioning and estrogen on apoE levels in the olfactory bulb revealed that estrogen is required for upregulation of apoE levels. Estrogen also influences synaptic sprouting in the dentate gyrus in response to injury via an apoE-dependent mechanism (Stone, 1998). Regions of the hippocampus and hypothalamus both showed synaptic remodeling during the estrous cycle and, apoE mRNA levels fluctuated according to the stages. In proestrus when E2 levels are elevated apoE mRNA levels are at a maximum. However, 24 hours later in estrus apoE mRNA levels are 30% less in the hypothalamus and CA1 region of the hippocampus (Stone, 97). Changes in synaptic density in the CA1 region of the hippocampus during the estrus cycle showed the same pattern as apoE mRNA levels with the highest synaptic density corresponding to proestrus, which is the peak of estrogen, (Woolley, 1992). Differential distribution and regulation of estrogen receptors throughout the brain (Osterlund, 1998) is presumed to be the reason for differences in the effects of estrogen on ApoE. By using the estrus cycle and ovariectomies plus pellet placement as my models I was able to examine the effects of estrogen on apoE levels throughout various brain regions. Male mice were used as a baseline in this study.

Statistically significant differences were found among brain regions for the concentration of apoE in the female mice. Evaluation of the male mice paralleled these data although these data did not reach significance. OB showed the highest concentration of apoE and cerebellum showed the lowest; cortical areas and hippocampus results fell between. The data show substantial regional and temporal variation (3-10 fold) during the estrous cycle. General statements about apoE protein concentration in female mice

require careful consideration of both of these factors in interpretation of endocrine effects on brain apoE.

Striatal concentrations of apoE were not affected by either estrous phases or E2 replacement. ER mRNA has not been reported in the rat striatum (Shrugrue et al, 1999, 2000, 2001). Estrous cycle neurotransmitter variation in the striatum may represent ER present in the substantia nigra, the source of striatal dopamine (Jori et al, 1976, Kritzer, 1997).

Regional variation of ER subtypes may underlay regional variation in apoE expression during the estrous cycle (Shrugrue et al, 1999, 2000, 2001). These studies reported neocortical areas and hippocampus expressed message for both ERα and β in rats. The olfactory bulb and cerebellum only expressed the β subtype of the ER. The striatum expressed neither. I suggest that E2 affects cortical apoE by the ERα. Activation by ERα could account for increasing concentration of apoE in diestrus and proestrus when E2 levels were increasing. Increased apoE could also be through a non-genomic mechanism as has been reported for some actions of E2 (Toran-Allerand et al, 1999; McEwen et al, 2001). In the olfactory bulb and cerebellum, which do not express the ERα in the rat, E2 stimulation of apoE is by the ERβ. Elevation may require prolonged exposure to a higher threshold of E2 or an intervening step of glial activation (McEwen, 2001), given that there is an estrogen response element in the GFAP (glial fibrillary acidic protein) promoter region (Stone, 1998). In this context, it should be noted that my evaluation of the promoter sequence of the mouse apoE gene revealed a region with significant similarity to the estrogen response element at 547 bases upstream from the transcriptional initiation site. Glial activation and this promoter may interact for apoE production.

The OVX-E2 replacement experiments generally paralleled the estrous cycle pattern of apoE expression and lend support to our speculation about the possible receptor function. The striatum showed no evidence of apoE changes with estrous cycle and was unresponsive to E2 replacement or deprivation. A significant effect was found in the areas reported to express only the β receptor (olfactory bulb and cerebellum). Significant effects were not found in the brain regions that express message for both α and β receptors (cortex and hippocampus). This observation is in contrast to a previous paper (Levin-Allerhand, 2001) that found an effect in cortex and hippocampus and attributed this change to the ERβ. This study used a much higher dosage of E2 (Levin-Allerhand, 2001). The pellets we used maintain circulating levels of E2 between 150-200 pg/ml for about eight weeks and literature values for proestrus levels vary between 40-100pg/ml (Rozosky, 2002). Sampling five days after E2 replacement/deprivation puts our sampling period close to maximal apoE elevation during normal estrous cycling in the olfactory bulb and cerebellum (i.e., estrus). Failure to find an E2 effect in areas expressing both ERα and β may be an artifact of timing. Sampling at 5 days after deprivation or replacement could mask an E2 effect modulated via ERα because the peak activation of apoE may have been passed. Pharmacological doses of E2 may be able to override homeostatic mechanisms.

Understanding the relationship between estrogen and apoE is clinically relevant. Hormone replacement therapy (HRT) decreases the risk for developing dementia (Brenner, 1994; Tang, 1996; Paganini-Hill, 1994), is associated with a later onset and less malignant course of Parkinson's disease (Kuopio, 1999; Saunder-Pullman, 1999), and decreases the risk or improves prognosis following stroke (Paganini-Hill, 1995).

Increased synaptic sprouting in response to estrogen requires apoE (Stone, 1998). We speculated previously that apoE may be involved in repair and recycling of membrane materials (Nathan, 2001). Because apoE is intimately involved in utilization of cholesterol and membrane integrity in the CNS, HRT could function by changing levels of apoE which, in turn, could enhance cholesterol utilization. Protective effects of HRT could be acting through an apoE-based mechanism.

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